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Activation of ERK accelerates repair of renal tubular epithelial cells, whereas it inhibits progression of fibrosis following ischemia/reperfusion injury

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ABSTRACT

Extracellular signal-regulated kinase (ERK) signals play important roles in cell death and survival. However, the role of ERK in the repair process after injury remains to be defined in the kidney. Here, we investigated the role of ERK in proliferation and differentiation of tubular epithelial cells, and proliferation of interstitial cells following ischemia/reperfusion (I/R) injury in the mouse kidney. Mice were subjected to 30 min of renal ischemia. Some mice were administered with U0126, a specific upstream inhibitor of ERK, daily during the recovery phase, beginning at 1 day after ischemia until sacrifice. I/R caused severe tubular cell damage and functional loss in the kidney. Nine days after ischemia, the kidney was restored functionally with a partial restoration of damaged tubules and expansion of fibrotic lesions. ERK was activated by I/R and the activated ERK was sustained for 9 days. U0126 inhibited the proliferation, basolateral relocation of Na,K-ATPase and lengthening of primary cilia in tubular epithelial cells, whereas it enhanced the proliferation of interstitial cells and accumulation of extracellular matrix. Furthermore, U0126 elevated the expression of cell cycle arrest-related proteins, p21 and phosphorylated-chk2 in the post-ischemic kidney. U0126 mitigated the post-I/R increase of Sec10 which is a crucial component of exocyst complex and an important factor in ciliogenesis and tubulogenesis. U0126 also enhanced the expression of fibrosis-related proteins, TGF- β 1 and phosphorylated NF- κ B after ischemia. Our findings demonstrate that activation of ERK is required for both the restoration of damaged tubular epithelial cells and the inhibition of fibrosis progression following injury.

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1. Introduction

Acute kidney injury (AKI) is a significant world-wide problem [1]. Mortality and morbidity by AKI in critically ill patients exceed 50% in some studies and these rates have not declined over the last several decades [2,3]. Ischemia/reperfusion (I/R) injury is one of the most common causes of AKI and occurs in many clinical settings including kidney transplantation and cardiac bypass surgeries [4]. The kidney is composed of many types of cells, including epithelial cells in the tubules and fibroblasts in the interstitium. Depending on the cell type and the time period following injury, cells display diverse pathological changes including: loss of cell polarity, disruption of cell to cell contacts, death, proliferation, dedifferentiation, accumulation of extracellular matrix (ECM), and proliferation of interstitial cells [5]. In a recent study, we found that the kidney responded differently

to antioxidant treatment depending on the starting time and duration of treatment, as well as the cell-type, resulting in differential consequences with respect to repair following I/R injury [6].

Mitogen-activated protein kinases (MAPK), including p38, c-Jun NH2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) play an important role in the survival and death in cells [7–9]. In a previous study, we found that I/R dramatically increased the phosphorylated ERK level in the kidney and the increased ERK level was sustained until 9 days later, when renal function, but not morphology, returned to approximately baseline [10]. This study suggests that ERK activation in the recovery period may be associated not only with the process of restoration of damaged tubular epithelial cells, but also increases in interstitial fibroblasts [10]. Furthermore, it suggests that the ERK signal may act cell-specifically and in a time-dependent manner in the repair following injury. However, the in vivo direct role of ERK remains to be defined in the recovery process following kidney I/R injury.

In the present study we investigated the role of ERK in tubular epithelial cell restoration and fibrotic changes following I/R injury in mice including: proliferation, primary ciliogenesis, polarity of tubular epithelial cells, proliferation of interstitial cells, and interstitial

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ECM accumulation. Here, we report that prevention of ERK activation by U0126, a specific inhibitor of MEK, which is directly upstream of ERK, retarded restoration of tubular epithelial cells, and accelerated fibrosis via increases in interstitial cell proliferation and ECM accumulation. These findings demonstrate for the first time *in vivo* that ERK contributes to the recovery following ischemic injury in the mouse kidneys, suggesting that the modulation of ERK may be therapeutically useful for the treatment of acute kidney injury and progression to fibrosis.

2. Materials and methods

2.1. Animal preparation

All experiments were conducted using 8-week-old C57BL/6 male mice weighing 20 to 25 g (Koatech, Gyeonggi-do, Korea). The studies were approved by the Institutional Animal Care and Use Committee of Kyungpook National University. Mice were permitted free access to water and standard mouse chow. The animals were anesthetized with pentobarbital sodium (60 mg/kg body weight; Sigma; St. Louis, MO) prior to surgery. Kidney ischemia was induced as described previously [10]. In brief, kidneys were exposed via flank incisions. The mice were subjected either to 30 min of bilateral or unilateral renal ischemia with non-traumatic microaneurysm clamps (Roboz Surgical Instruments; Washington D.C.). The incisions were closed temporarily during ischemia. After the removal of the clamps, reperfusion was visually confirmed. Body temperature was maintained at 36.5–37.5 °C during the operation. Some of the mice received intra-peritoneal administration of U0126 (10 mg/kg BW; Selleckchem, Houston, TX) or vehicle beginning at 24 h after I/R or sham until 24 h prior to harvest daily. To evaluate the accumulation of proliferating cell in the kidney, mice were treated with 5-bromo-2-deoxyuridine (BrdU, 50 mg/kg body weight; Sigma, St. Louis, MO) beginning at 24 h after I/R or sham until 24 h prior to harvest daily [11]. Sham surgery was equivalent to the operation for ischemia except for clamping of the renal pedicle. To perform biochemical and histological experiments, kidneys were snap-frozen in liquid nitrogen and perfusion-fixed with 30 mL of phosphate buffered saline (PBS) for 2 min and then PLP (4% paraformaldehyde, 75 mM L-lysine, 10 mM sodium periodate; Sigma) solution, respectively. Frozen tissue was kept in the deep-freezer until use.

2.2. Plasma creatinine concentration

The concentrations of plasma creatinine were measured using a Vitros250 (Johnson and Johnson) [12].

2.3. Histology

Perfusion-fixed kidneys were excised and placed in PLP overnight at 4 °C. Tissue samples were then cut into 4- μ m paraffin-embedded sections using a microtome (Leica, Bensheim, Germany). Kidney-sections were stained with periodic acid Schiff (PAS) or Masson trichrome according to a standard protocol. Collagen deposition was quantified using the i-solution DT image acquisition and analysis program (iMT i-solution, Vancouver, Canada), incorporating a Nikon Fx35 (Nikon, Japan) in 10 fields in the outer medulla per kidney [13]. Morphological damage levels in PAS-stained kidney section were scored as described previously [14]. Briefly, fifty tubules in the outer medullary region of the kidney were analyzed using a score of 0, no damage; 1, mild damage with rounding of epithelial cells and a dilated tubular lumen; 2, severe damage with flattened epithelial cells, loss of nuclear staining, a dilated lumen, and congestion of the lumen; and 3, destroyed tubules with flat epithelial cells lacking nuclear staining and congestion of the lumen. At least four kidneys from each experimental condition were used. Ten fields per slide were used for the counting. Pictures were taken in the outer medulla.

2.4. Measurement of primary cilia length

Kidney sections were processed for immunofluorescence microscopy by staining with anti-acetylated α -tubulin antibody and DAPI as described above. Images were captured using Axioplan-2 epifluorescence microscope (Carl Zeiss, Thornwood, NY). 5–10 fields in the cortex were randomly captured (400 \times) and measured in each segment per time point from 3 independent animals. More than 50 cells from each experiment were used to measure cilia length. iSolution (iMT i-solution, Vancouver, Canada) software was used to trace and measure the length of cilia in captured images.

2.5. Western blot

Western blot analyses were performed as described previously [12]. Antibodies used were; phospho-ERK (Cell Signaling Technology, Danvers, MA), total-ERK (Cell Signaling Technology, Danvers, MA), collagen I (Santa Cruz Biotechnology, Santa Cruz, CA), α -SMA (Sigma, St. Louis, MO), Na,K-ATPase [15], Sec10 [16], TGF- β 1 (Santa Cruz Biotechnology, Santa Cruz, CA), fibronectin (Santa Cruz Biotechnology, Santa Cruz, CA), p-NF- κ B (Cell Signaling Technology, Danvers, MA), Ly6G (eBioscience, San Diego, CA), p21 (Santa Cruz Biotechnology, Santa Cruz, CA), p-chk2 (Cell Signaling Technology, Danvers, MA) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) as a marker for protein loading. LabWorks software (UVP, Upland, CA) was used for quantification of band densities.

2.6. Immunofluorescence and immunohistochemistry staining

Immunofluorescence and immunohistochemistry staining were performed as described previously [13,17]. Antibodies used were: BrdU (Serotec, Oxford, UK), acetylated tubulin (Sigma, St. Louis, MO), Na,K-ATPase [15,18], FSP-1 (Novus, Littleton, CO) and p21 (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were observed under an LSM 5 confocal microscope (Carl Zeiss, Thornwood, NY) or an Axioplan-2 epifluorescence microscope (Carl Zeiss, Thornwood, NY) or Nikon Fx35 (Nikon, Japan). Pictures were taken in the outer medulla.

2.7. Statistics

Results were expressed as the means \pm SEM. Statistical differences among groups were calculated using Student's *t*-test. Each experimental group consisted of at least three mice. Differences between groups were considered statistically significant at a *P* value of <0.05.

3. Results

3.1. Increased plasma creatinine levels after I/R gradually return to the normal range, whereas fibrotic lesion is expanded over time

Consistent with our previous studies [6,10,12,15], the concentration of plasma creatinine (PCr) was significantly elevated 24 h following 30 min of bilateral renal ischemia (Fig. 1A). The increased PCr returned to approximately normal ranges by post-ischemic day 9 (Fig. 1A). Kidneys exposed to 30 min of ischemia in mice had disruption and loss of brush border of tubular epithelial cells with atrophy, dilation, and congestion of tubules (Fig. 1B). The tubular damage score peaked at 24 h after ischemia and then gradually decreased over time, whereas the fibrotic lesions gradually increased over the time period (Fig. 1B and C). Collagen deposition and α -SMA expression significantly increased in the kidneys exposed to ischemia, and these increases were gradual over time (Fig. 1C and D). Fibronectin is associated with the process of repair, including fibrosis [19–21]. The expression of fibronectin gradually increased

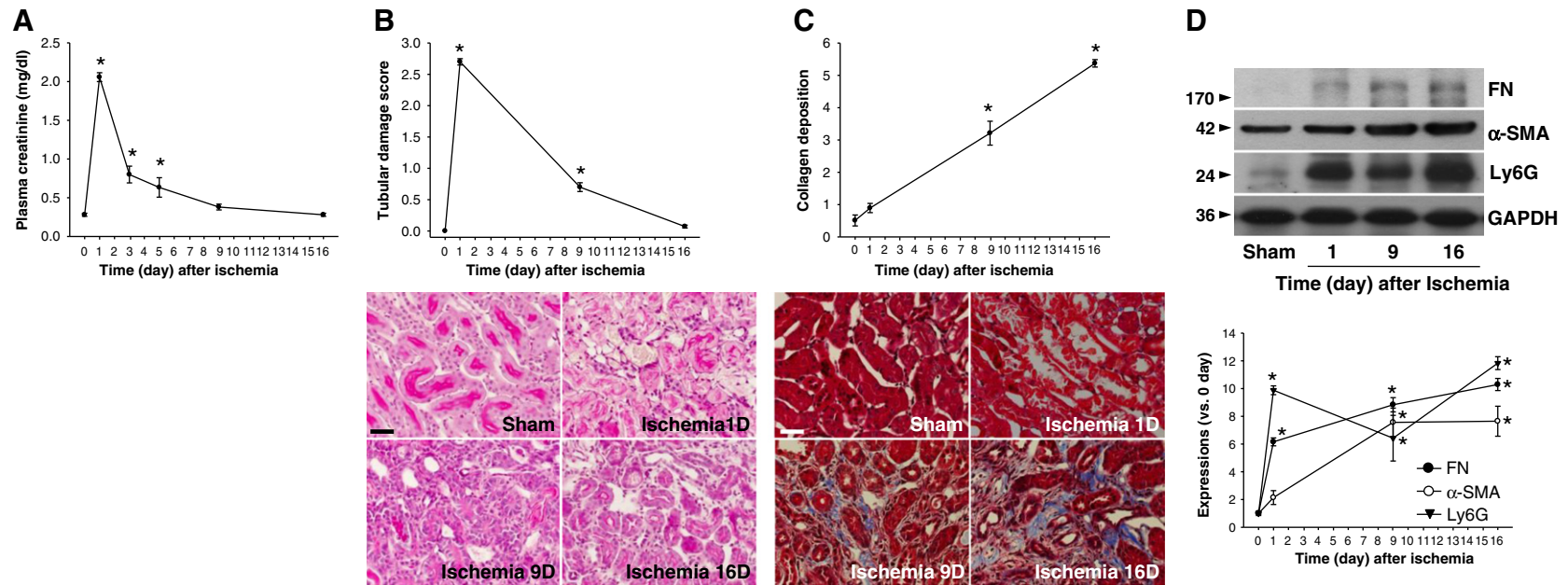


Fig. 1. Time course of plasma creatinine, tubular damage, and collagen deposition after ischemic injury in the kidney. Mice were subjected to either 30 min of bilateral renal ischemia (Ischemia) or sham surgery (Sham). (A) Plasma creatinine concentrations were measured at the indicated time points. (B) Tubular damage score and (C) collagen deposition were measured at the indicated time points. The evaluation of tubular damage and collagen deposition involved averaging the values from 10 fields per kidney. (B, C) Kidneys were harvested at the indicated time points after the operations and examined with periodic acid-Schiff and trichrome staining as described in the [Materials and methods](#) section. Collagen deposition by Masson's trichrome staining is shown by the blue color. Pictures were obtained from the outer medulla. PAS- and trichrome-stained images were representative of the indicated time points. (D) Western blot was performed using antibodies against fibronectin, α -SMA, and Ly6G. The density of the bands was quantified using LabWorks analysis software. GAPDH was used as a loading control. FN = fibronectin. Scale bar indicates 50 μ m. The results are expressed as the means \pm SEM ($n = 4-6$). *, $P < 0.05$ vs. sham.

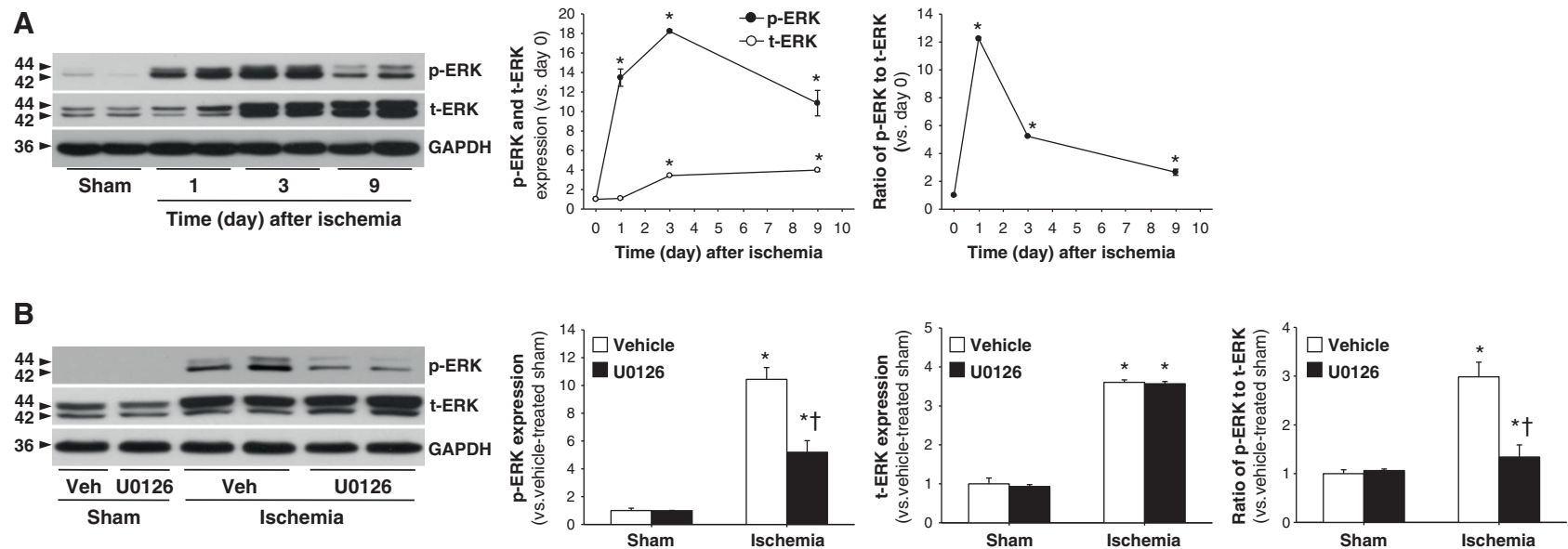


Fig. 2. Sustained activation of ERK after ischemic injury in the kidney. Mice were subjected to either 30 min of unilateral renal ischemia (Ischemia) or sham surgery (Sham). Some mice received U0126, an inhibitor of MEK, daily from 24 h after the operation to 24 h prior to the harvest. Kidneys were harvested at the indicated time points, or 9 days after the operations, and western blot analysis was performed as described in the [Materials and methods](#) section. (A and B) Western blot bands, shown in duplicate, were representative for the indicated time points. The densities of phosphorylated-ERK (p-ERK) and total-ERK (t-ERK) were quantified using LabWorks analysis software. GAPDH was used as a loading control. Veh = vehicle. The results were expressed as the means \pm SEM ($n = 5$). *, $P < 0.05$ vs. sham; †, $P < 0.05$ vs. vehicle-treated ischemia.

over time (Fig. 1D). The infiltration of neutrophils into the kidney markedly increased at early time-points and this increase was sustained for longer than 16 days (Fig. 1D).

3.2. ERK activated by ischemia is present for a long period of time

To minimize the effect of uremia on ERK activation, we determined ERK activation in a mouse model of unilateral ischemia, which does not develop uremia [10]. 30 min of unilateral renal ischemia dramatically increased active phosphorylated ERK (p-ERK) levels (Fig. 2A). The increase of p-ERK peaked at 3 days after ischemia and gradually decreased over time. However, the increased p-ERK level did not return to normal levels until 9 days after ischemia (Fig. 2A). Total ERK levels increased gradually until 9 days after ischemia (Fig. 2A). The ratio of p-ERK to t-ERK in the I/R-injured kidneys was higher than that in the sham-operated kidneys (Fig. 2A). Treatment with U0126, a specific inhibitor of MEK, beginning at 24 h after ischemia daily, significantly prevented the increase of p-ERK, but not t-ERK (Fig. 2B).

3.3. Inhibition of ERK activation retards restoration of damaged tubules, whereas it enhances fibrosis

Nine days after ischemia, tubular atrophy, dilation, and congestion of tubules were significantly greater in the U0126-treated mouse kidneys than in the vehicle-treated mouse kidneys (Fig. 3A). The number of interstitial cells also was greater in the U0126-treated mouse kidneys when compared with that in the vehicle-treated mouse kidneys (Fig. 3A). Consistent with the morphological evaluation, tubular damage scores were significantly higher in the U0126-treated mouse

kidneys than in the vehicle-treated mouse kidneys (Fig. 3A). Collagen deposition in the U0126-treated mouse kidneys was also much greater than in the vehicle-treated mouse kidneys 9 days following ischemia (Fig. 3B).

3.4. Inhibition of ERK activation decreases proliferation of tubular epithelial cells, whereas it accelerates proliferation of interstitial cells

To detect proliferating cells in the kidney, we administered BrdU daily into mice beginning at 24 h after ischemia until sacrifice. BrdU-positive cells in the tubules and interstitium of the sham-operated mouse kidney were rarely observed (Fig. 4A and B). On the other hand, 9 days after ischemia, the numbers of BrdU-positive cells were significantly increased in both the tubules and the interstitium when compared with the sham-operated kidneys (Fig. 4A and B). The post-ischemic increase of BrdU-positive cells in the kidney tubules was significantly less in the U0126-treated mouse kidneys than in the vehicle-treated kidneys (Fig. 4A). As opposed to the results in tubules, the number of BrdU-positive cells in the interstitium was greater in the kidneys of U0126-treated mice than that of vehicle-treated mice 9 days after I/R (Fig. 4B). Expression of p21 and p-chk2 induces cell cycle arrest, G1/S or G2/M arrest, which is associated with kidney fibrosis progression [22–26]. Post-ischemic expression of p21 and p-chk2 was highly upregulated in the post-ischemic kidneys, compared to the sham-operated kidneys (Fig. 4C). Moreover, tubular localization of p21 was also increased in the post-ischemic kidneys, and its localization was greater in the U0126-treated I/R-injured kidneys than that in the vehicle-treated kidneys (Fig. 4D), suggesting that the deterioration of

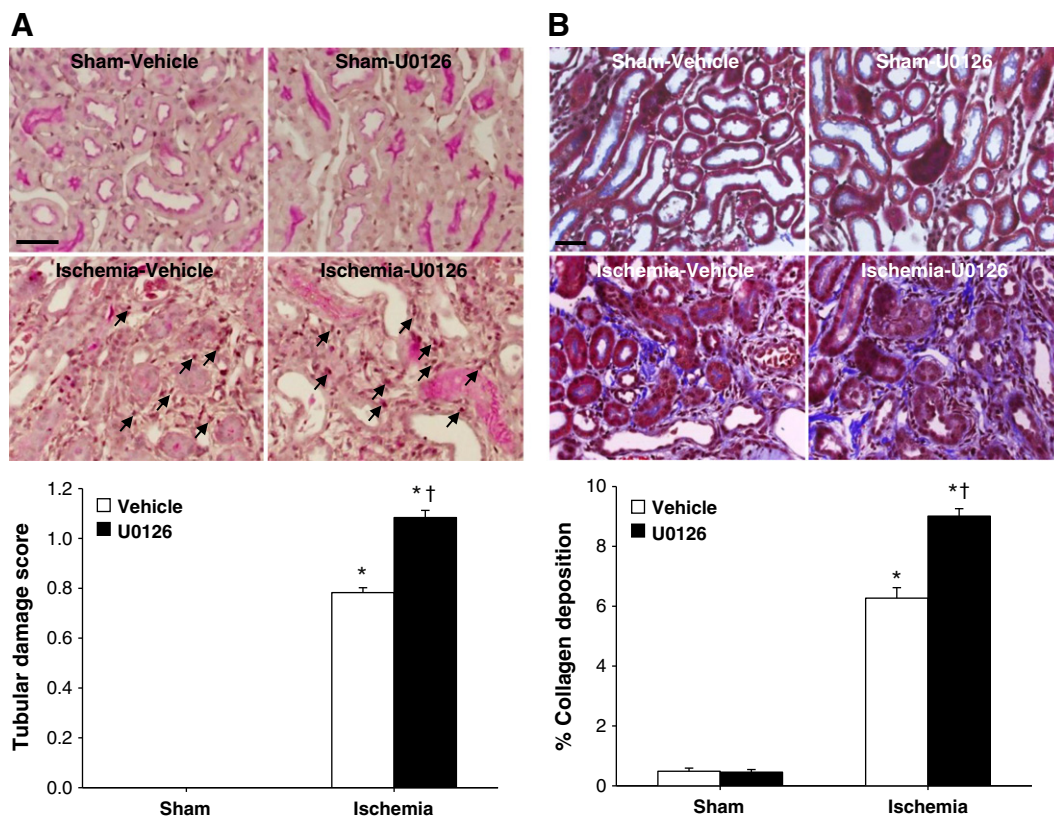


Fig. 3. Delayed epithelial cell recovery and accelerated progression of fibrosis by inhibition of ERK. Mice were subjected to either 30 min of unilateral renal ischemia (Ischemia) or sham surgery (Sham). Kidneys were harvested at 9 days following ischemia. Some mice received U0126, an inhibitor of MEK, daily from 24 h after the operation to 24 h prior to the harvest. (A) To evaluate tubular damage, kidney sections were stained with periodic acid-Schiff reagent as described in the Materials and methods section. Pictures were obtained from the outer medulla. The score of tubular damage was a result of averaging the values from 10 fields per kidney. (B) To evaluate collagen deposition, kidney sections were exposed to trichrome staining as described in the Materials and methods section. Collagen deposition by Masson's trichrome staining is shown by the blue color. Pictures were obtained from the outer medulla. The area of collagen deposition was determined by averaging the values from 10 fields per kidney. Arrow indicates the interstitial cell. Scale bar indicates 50 μ m. The results were expressed as the means \pm SEM ($n = 5$). *, $P < 0.05$ vs. sham; †, $P < 0.05$ vs. vehicle-treated ischemia.

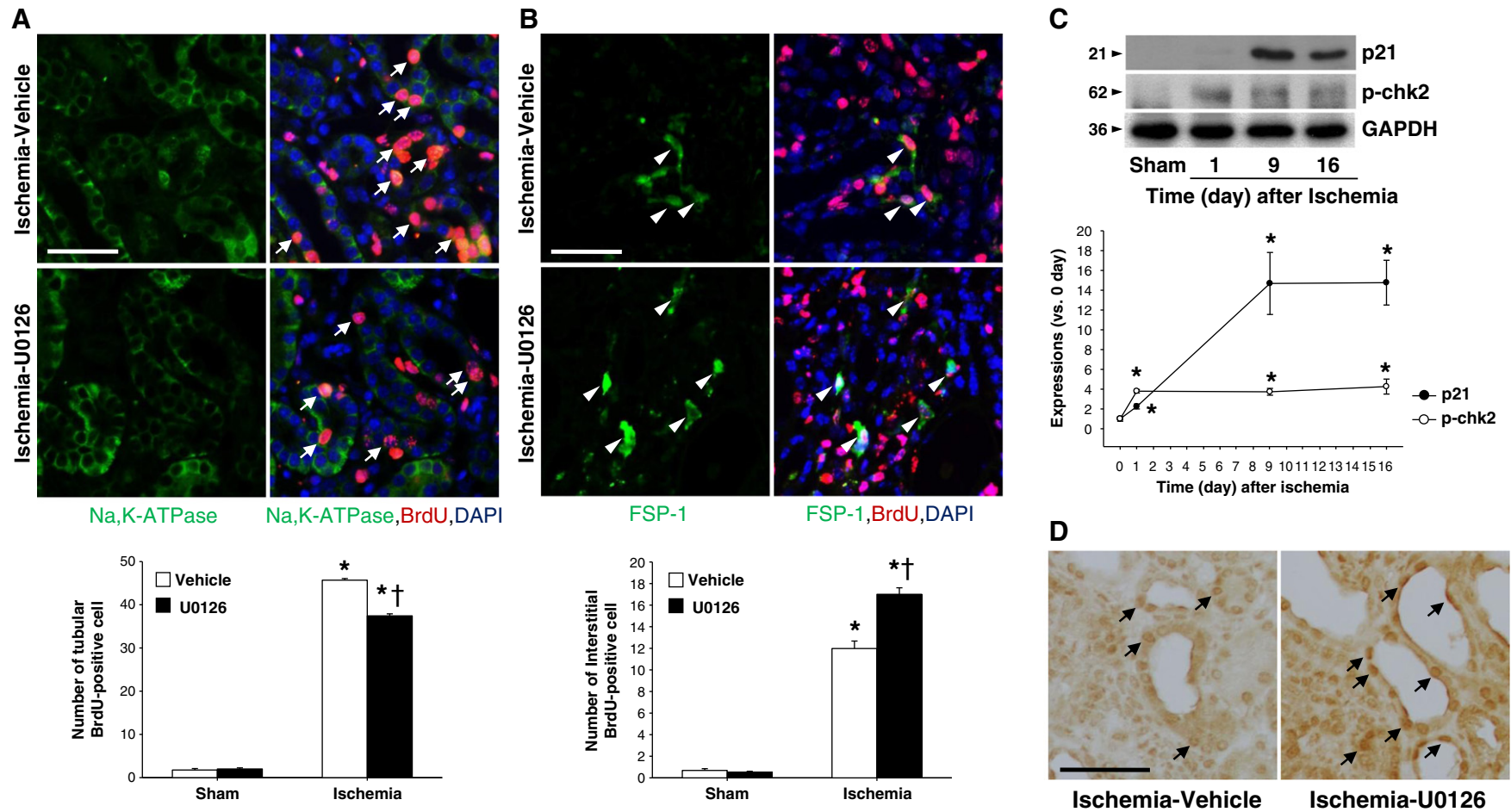


Fig. 4. Effect of inhibition of ERK in the cell proliferation of ischemic kidneys. Mice were subjected to either 30 min of unilateral renal ischemia (Ischemia) or sham surgery (Sham). Some mice received U0126, an inhibitor of MEK, daily from 24 h after the operation to 24 h prior to harvest. To detect (A) tubular and (B) interstitial cell proliferation after ischemic injury, BrdU was administered to mice daily from 24 h after the operation to 24 h prior to harvest. Kidneys were harvested at 1, 9 and 16 days following ischemia. Kidneys were immunofluorescence-stained using antibodies against Na,K-ATPase, FSP-1, and BrdU as described in the [Materials and methods](#) section. Pictures were obtained from the outer medulla. (A) The green, red and blue color indicates Na,K-ATPase, BrdU-positive cells, and DAPI-stained nuclei, respectively. (B) The green, red and blue color indicates FSP-1, BrdU-positive cells, and DAPI-stained nuclei, respectively. The number of BrdU-positive cells was an average of the values from 10 fields of tubules and interstitium per kidney. Arrow and arrowhead indicate tubular and interstitial BrdU-positive cells, respectively. Scale bar indicates 50 μ m. (C) Western blot analysis was performed using antibodies against p21 and p-chk2 as described in the [Materials and methods](#) section. Western blot bands were representative for the indicated time points. The densities of p21 and p-chk2 were quantified using LabWorks analysis software. GAPDH was used as a loading control. (D) Kidneys were immunohistochemically stained using an antibody against p21 as described in the [Materials and methods](#) section. Pictures were obtained from the outer medulla. Images were representative of each group. Arrow indicates p21-positive nucleus. Scale bar indicates 50 μ m. The results were expressed as the means \pm SEM ($n = 4-6$). *, $P < 0.05$ vs. sham; †, $P < 0.05$ vs. vehicle-treated ischemia.

fibrosis progression in the post-ischemic kidneys caused by U0126 treatment is related to tubular cell cycle arrest.

3.5. Inhibition of ERK activation retards basolateral relocalization of Na, K-ATPase, lengthening of primary cilia, and increase of Sec10 expression after I/R

Expression of Na/K-ATPase, which is an important membrane transporter and is localized on the basolateral membrane, is a critical factor for the restoration of polarity in the tubular epithelial cell [27]. Nine days after ischemia, the amount of Na,K-ATPase expression in the I/R-injured kidney was significantly decreased compared with that in the non-I/R-injured kidney and the decrease was much greater in the U0126-treated mouse kidneys than that in the vehicle-treated mouse kidneys (Fig. 5A). In the sham-operated kidneys, Na,K-ATPase was localized basolaterally in a stripe pattern in proximal tubular epithelial cells (Fig. 5B). U0126-treatment in the sham-operated kidney did not affect the distribution (Fig. 5B). In the ischemic injured kidneys, Na,K-ATPase was much fainter and distributed over the entire cell membrane of proximal tubular epithelial cells (Fig. 5B). The change of Na,K-ATPase expression after I/R was much greater in the U0126-treated compared with the vehicle-treated mouse kidneys (Fig. 5).

Primary cilia protruded from the apical surface into the lumen (Fig. 6A). Primary cilia are shortened or disappear in the injury period following I/R and then lengthen during the recovery period [28]. Nine days after ischemia, primary cilia lengths were significantly longer when compared with those in the sham-operated control kidney (Fig. 6A). Primary cilia lengths in the U0126-treated mouse kidneys were significantly shorter than those in vehicle-treated mouse kidneys 9 days after ischemia (Fig. 6A). Sec10, a crucial component of the exocyst complex, accelerates the recovery of damaged epithelial cells and regulates the length of primary cilia via ERK activation [29]. I/R increased the expression of Sec10 in the kidney, but the increase in Sec10 expression was significantly less in the U0126-treated mouse kidneys than in the vehicle-treated mouse kidneys (Fig. 6B).

3.6. Inhibition of ERK increases expression of TGF- β 1, activated-NF- κ B, and fibrosis marker proteins in the kidney

Nine days after ischemia, the expression of TGF- β 1 in the kidney was significantly greater than that found in the sham-operated kidneys (Fig. 7A and B). The post-ischemic increase of TGF- β 1 in the U0126-treated mice was significantly greater than in the vehicle-treated mouse (Fig. 7A and B). Expression of collagen I, fibronectin, and α -SMA in the kidney subjected to ischemia was greater than that in the kidney subjected to sham-operation (Fig. 7A and C–E). U0126 treatment further enhanced the post-ischemic increase of collagen I, fibronectin and α -SMA (Fig. 7A and C–E). Ischemia increased the phosphorylation of NF- κ B (Fig. 7A and F) and the increase of phosphorylation of NF- κ B was significantly enhanced by U0126 treatment (Fig. 7A and F). Consistent with the activation of NF- κ B, the expression of Ly6G was greater in the U0126-treated ischemic kidneys than it was in the vehicle-treated ischemic kidneys (Fig. 7A and G).

4. Discussion

In the present study, our major findings are that 1) ERK activated by I/R injury lasted for a lengthy period of time following ischemia, 2) U0126 treatment during the recovery period retarded the restoration of damaged tubular epithelial cells, whereas it accelerated the progression of fibrosis, and 3) U0126 treatment inhibited the lengthening of primary cilium, with the prevention of a post-ischemic ischemic increase in exocyst Sec10 expression. Based on our findings, we conclude that activated ERK during recovery plays a key role in both the restoration of damaged tubular epithelial cells and the inhibition of fibrosis progression.

Tubular epithelial cells have a great capacity for recovery and replenishment of damaged epithelial cells through proliferation and differentiation of surviving epithelial cells, under the regulation of a number of intrinsic temporally-expressed cellular signals [30–32]. ERK has long been considered as a main modulator of kidney I/R injury by regulating survival and death in the kidney, depending on the severity of injury and treatment [10,15,33]. However, the direct role of ERK in the repair after I/R injury in the kidney remains to be defined. In the

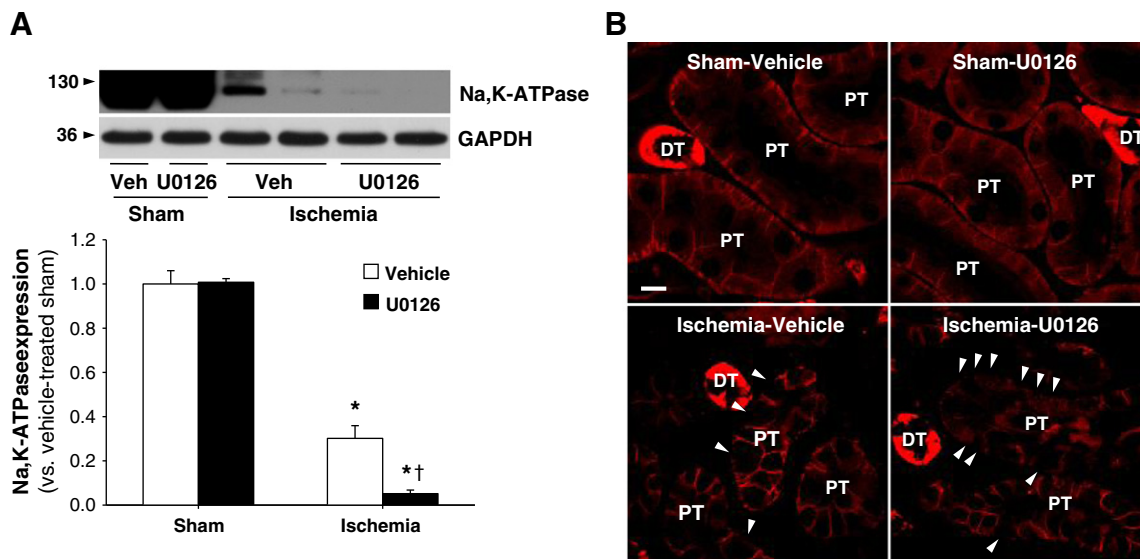


Fig. 5. Effect of inhibition of ERK in the expression of Na,K-ATPase in ischemic kidneys. Mice were subjected to either 30 min of unilateral renal ischemia (Ischemia) or sham surgery (Sham). Some mice received U0126, an inhibitor of MEK, daily from 24 h after the operation to 24 h prior to harvest. Kidneys were harvested at 9 days following ischemia. (A) Western blot was performed using an anti-Na,K-ATPase antibody. The density of the bands was quantified using LabWorks analysis software. GAPDH was used as a loading control. (B) Kidney sections were stained using an antibody against Na,K-ATPase as described in the Materials and methods section. Na,K-ATPase-positive areas are shown in red. Pictures were obtained from the outer medulla and were representative of three individual experiments. Arrowhead indicates absent or mislocalized Na,K-ATPase. Veh = vehicle; DT = distal tubule; PT = proximal tubule. Scale bar indicates 10 μ m. The results were expressed as the means \pm SEM ($n = 5$). *, $P < 0.05$ vs. sham; †, $P < 0.05$ vs. vehicle-treated ischemia.

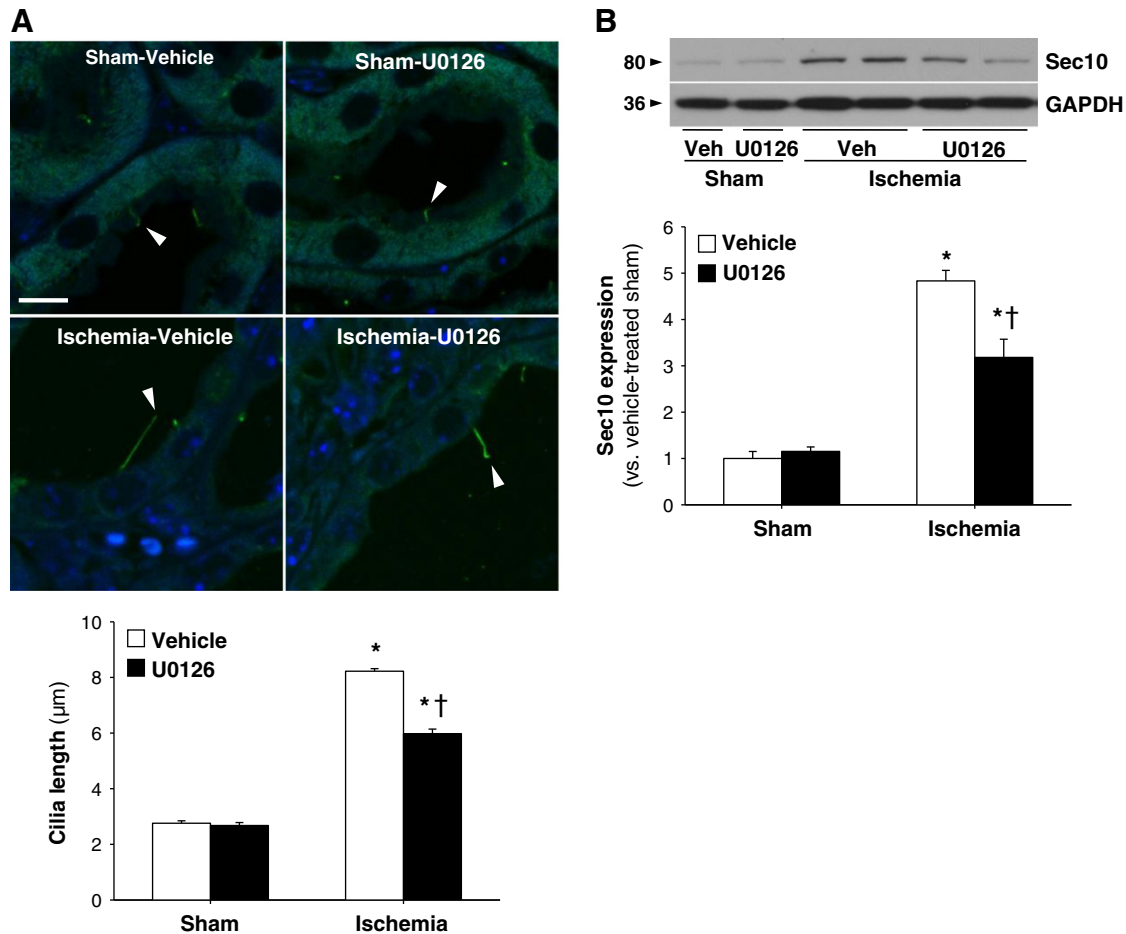


Fig. 6. Effect of inhibition of ERK on primary cilia length and Sec10 expression in ischemic kidneys. Mice were subjected to either 30 min of unilateral renal ischemia (Ischemia) or sham surgery (Sham). Some mice received U0126, an inhibitor of MEK, daily from 24 h after the operation to 24 h prior to harvest. Kidneys were harvested at 9 days following ischemia. Kidney sections were stained using an antibody against acetylated alpha tubulin as described in the Materials and methods section. (A) Acetylated alpha tubulin-positive cilia are shown in green. The blue color represents DAPI-stained nuclei. Pictures were obtained from the outer medulla. Scale bar indicates 10 μm. The length of acetylated alpha tubulin-positive cilia in the graph was an average of the values from 50 cilia in proximal tubules per kidney. Arrowhead indicates primary cilia. (B) Western blot was performed using an antibody against Sec10. The density of the Sec10 band was quantified using LabWorks analysis software. GAPDH was used as the equal loading marker. Veh = vehicle. The results were expressed as the means ± SEM (n = 5). *, $P < 0.05$ vs. sham; †, $P < 0.05$ vs. vehicle-treated ischemia.

present study, we found that I/R increased the activation and amount of ERK, and these increases lasted until the kidney was functionally restored. Several previous studies reported that chronic kidney injury, including ischemic injury, activates ERK [34–36]. We also found that the blockage of ERK activation using U0126 delayed the recovery of tubular epithelial cells, and accelerated progression of fibrosis. These data indicate that ERK may act in a cell-specific manner in the kidney during recovery following injury.

Recent studies have demonstrated that the ERK signaling pathway is involved in anti-inflammatory and anti-oxidant effects in diverse cells [37–40]. Maeng et al. reported that ERK directly inhibits NF-κB signaling, thereby suppressing the inflammation in endothelial cells, which is critical for the regulation of inflammatory cells recruited into damaged tissue [41]. In addition, we and others have reported that ERK improves the survival of kidney epithelial cells following ischemia/reperfusion injury, and oxidant injury is associated with progression of fibrosis [10,13,33]. In the present study we found that ERK inhibition accelerated leukocyte infiltration in the injured kidney with increased activation of NF-κB. Therefore, the role of ERK in acute and chronic kidney injury may be associated with the anti-inflammatory and anti-oxidative effects.

Jo et al. demonstrated that pretreatment with U0126 before an ischemic insult protects the kidney from ischemic injury [42]; however, in the present study U0126 treatment worsened kidney fibrosis. This may be due to the timing of U0126 treatment. Jo et al. treated U0126

mice before injury [42], whereas we administered U0126 after the peak of injury, i.e., during the recovery phase. This may account for the different results generated by Jo et al., and suggests that ERK acts differently with respect injury and repair depending upon the time of treatment and the cell type. Furthermore, recent reports have suggested an association between kidney fibrosis progression and cell cycle arrest [5,23,24]. Yang and colleagues reported that cell cycle arrest of acutely injured tubular epithelial cell worsens kidney fibrosis [25]. In the present study, expression of p21 and p-chk2, cell cycle arrest markers, increased in post-ischemic kidney and these increases were higher in the U0126-treated post-ischemic kidneys than in the vehicle-treated post-ischemic kidneys. This suggests that the worsened kidney fibrosis caused by ERK inhibition in the ischemic kidney, may be due, at least in part, to tubular cell cycle arrest.

Na,K-ATPase, one of the most prominent transmembrane proteins in the tubular epithelial cell, is predominantly expressed on the basolateral plasma membrane, and is a major contributor to the maintenance of cell polarity in the tubular epithelial cell [4,43]. The change in distribution of Na,K-ATPase is closely related to the injury and differentiation of cells. In previous studies, we found that the localization of Na,K-ATPase was severely disrupted after I/R injury; in post-ischemic kidneys the disruption was sustained for up to 8 days and then gradually recovered over time [29]. In the present study, the alteration of location and expression levels of Na,K-ATPase was similarly seen in the I/R injured kidney and these changes were greater in the U0126-treated

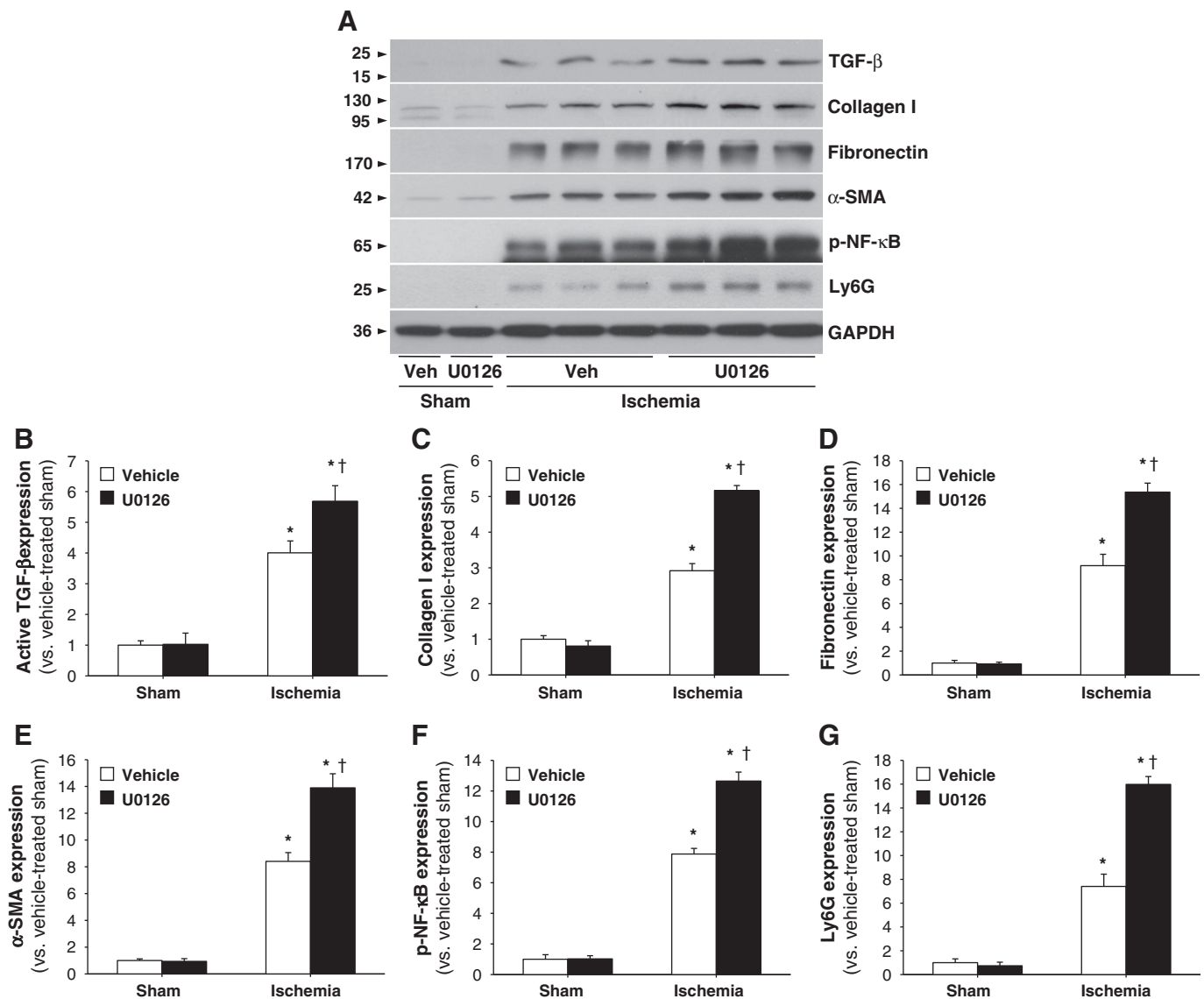


Fig. 7. Enhanced interstitial fibrosis of ischemic kidneys by inhibition of ERK. Mice were subjected to either 30 min of unilateral renal ischemia (Ischemia) or sham surgery (Sham). Kidneys were harvested at 9 days following ischemia. Some mice received U0126, an inhibitor of MEK, daily from 24 h after the operation to 24 h prior to harvest. Western blot was performed using antibodies against TGF- β , collagen I, fibronectin, α -SMA, -p-NF- κ B and Ly6G. The density of bands was quantified using LabWorks analysis software. GAPDH was used as a loading control. Veh = vehicle. The results were expressed as the means \pm SEM ($n = 5-6$). *, $P < 0.05$ vs. sham; †, $P < 0.05$ vs. vehicle-treated ischemia.

mouse kidneys than in the vehicle-treated mouse kidneys. These data indicate that ERK1/2 activation is required for the restoration of Na, K-ATPase both morphologically and quantitatively after I/R injury, indicating that ERK1/2 activation is necessary for both the restoration of cell polarity, disrupted by I/R injury, and for the prevention of undesirable features of wound healing, such as fibrosis. Recently Rajasekaran and colleagues reported that treatment with TGF- β 1, which is a well-known inducer of fibrosis, changes Na,K-ATPase localization and that the altered Na,K-ATPase localization is associated with progression of fibrosis [44].

Alterations of cilia length are found in diverse pathophysiological conditions including I/R and ureteral obstruction injury [28,45]. Verghese and colleagues reported that I/R injury in the kidneys induces alterations of primary cilia length in a temporal pattern; lengths of primary cilia are shortened early after I/R injury, lengthen during recovery, and finally return to a normal size. Further, they reported that the outcome of transplanted kidneys is associated with changes of primary cilia length [45]. In the present study, we observed that the length of primary cilia in the recovering tubular epithelial cells was increased. We also found that the length of primary cilia in

the U0126-treated mouse kidneys was significantly shorter than that in the vehicle-treated mouse kidneys, indicating that ERK signals regulate the reconstruction of primary cilia altered by I/R injury, and consequently lead to the restoration of damaged tubules. Recently, it has been demonstrated that the activation of the ERK pathway is necessary for primary ciliogenesis and tubulogenesis [29,46,47].

Accumulating in vitro evidence demonstrates that exocyst Sec10 mediates function in tubulo-, cysto-, and ciliogenesis [16,29,48,49]. Recently, we found that the overexpression of Sec10 can rescue kidney epithelial cells from the loss of epithelial barrier function induced by oxidative stress, and facilitate the recovery [29]. This rescue effect of Sec 10 overexpression was blocked by U0126 treatment [29]. In the present study, we found that the expression of Sec10 increased in the recovering kidney, and this increase is reduced by U0126 treatment. This suggests that the regulation of Sec10 by ERK contributes to the recovery process of acutely injured epithelial cells. In addition, it was reported that Sec10 overexpression increased the length of primary cilia [16,29,48,49]. Therefore, we conclude, based on all these results, that ERK activation is necessary for the regulation of primary cilia length and recovery following kidney injury.

Regardless of the origin of kidney interstitial fibroblasts, the local proliferation of interstitial fibroblasts is a major factor in kidney fibrosis [50]. Ponnusamy et al. reported that ERK mediates the death of interstitial fibroblasts [51]. In the present study, U0126 treatment accelerated fibroblast proliferation and ECM accumulation, whereas it inhibited the proliferation of tubular epithelial cells, indicating that ERK activation negatively regulates progression of fibrosis, and positively regulates the restoration of damaged tubular epithelial cells. Supporting this idea, Chen et al. reported delayed recovery from ischemic injury in the kidneys of proximal tubule-specific epidermal growth factor receptor (EGFR) knock-out mice, which is due to a decrease in ERK1/2 activation [38]. Moreover, disruption of primary cilia leads to fibrosis along with a deregulation of cell growth and polarity [52]. TGF- β signaling is critical to progression of kidney fibrosis in experimental and clinical chronic kidney disease [53]. Activation of NF- κ B by TGF- β contributes to fibrotic progression through the regulation of the inflammatory response, which is one of the mediators for fibrogenesis [54–56]. Our present study showed that inhibition of ERK results in an increase of TGF- β and activation of NF- κ B, indicating that enhanced signaling by these factors may worsen fibrotic progression in the kidneys of U0126-treated mice. Finally, we suggest that fibrotic changes in the kidney after I/R injury may be controlled by cell-type specific regulation of ERK activation.

Conflict of interest

The authors declare that they have no conflict of interest.

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